Supplementary Text

Screen Optimization

To test whether our magnetic separation strategy would accurately distinguish phagocytosing from non-phagocytosing cells, we attempted to separate differentiated U937 cells that had successfully taken up superparamagnetic beads with an anionic surface from bead-negative cells. First, to limit the possible effect of opsonins in the serum, U937 cells were exchanged into serum-free medium. Next, 1.3 μm diameter fluorescent superparamagnetic beads with an anionic surface were added, which were rapidly phagocytosed. After phagocytosis, cells were washed, trypsinized to remove surface-bound beads, lifted from the culture dish, and passed through a magnetized column. Magnet-bound cell fractions were highly-enriched for bead-associated cells, with even a single bead conferring sufficient magnetism to capture associated cells on the column (Fig. 1b). Bead-containing cells were extremely rare in the magnet-unbound cellular fraction, while bead-free cells were limited to <10% of the magnet-bound fraction, attesting to the high sensitivity and specificity of the selection. Importantly, cells treated with the actin polymerization inhibitor cytochalasin D during bead exposure were unable to accumulate beads and showed drastically reduced retention on magnetic columns (Supplementary Fig. 1f), demonstrating that column binding requires actin-dependent particle uptake and does not result merely from surface binding of particles. In the absence of identification of a defined receptor for bead uptake, we cannot rule out the possibility of macropinocytosis (not phagocytosis) as a possible mechanism for bead ingestion. Nonetheless, the recovery of similar hits across a range of bead sizes and limited magnetization of cells after exposure to IONPs (see below) suggest that our screens primarily reveal regulators of phagocytosis.

We sought to generalize this approach to enable identification of genes that contribute to phagocytosis of particles lacking inherent magnetism. One such substrate is myelin debris, which is not efficiently phagocytosed after central nervous system injury and acts as an impediment to axonal regeneration and functional recovery[1]. In contrast, myelin is rapidly cleared after peripheral nerve injury and is likewise efficiently phagocytosed by differentiated U937 cells, as evidenced by rapid actin-dependent accumulation of pHrodo-conjugated myelin (Supplementary Fig. 2a). To allow for magnetic selection of cells based on myelin phagocytosis, we covalently conjugated 30 nm diameter iron oxide nanoparticles (IONPs) to purified myelin. We performed a titration of IONP labeling densities to determine the minimal level conferring sufficient magnetism to allow for efficient capture of phagocytosing cells on magnetic columns. Importantly, magnetic capture of U937 cells exposed to IONP-conjugated myelin was abolished by cytochalasin D, and IONPs on their own do not appreciably magnetize U937 cells, even over several hours, arguing that magnetization indeed results from phagocytosis of myelin (Supplementary Fig. 2b).

We next tested whether a mixture of U937 cells with different phagocytic capacities could be reliably separated on the basis of varying degrees of IONP-conjugated myelin uptake. As uptake of the fluorescent, superparamagnetic myelin increases over time (Supplementary Fig. 2a), we simulated differing levels of phagocytosis by exposing cells to myelin for either 2 hours (less internalized myelin) or 6 hours (more internalized myelin). We then labeled the 2-hour population with calcein, mixed the two populations, and tested how cleanly they could be separated by magnetic sorting. We found that 90% of recovered cells from the 6 hour pool were captured on the magnet, whereas only 60% of cells from the 2 hour pool were retained, indicating that different extents of phagocytosis of superparamagnetic myelin can be distinguished at a population level using magnetic sorting (Supplementary Figs. 2c-f).

In these experiments, we could observe selective capture of highly phagocytic cells in the magnet-bound fraction by following enrichment of signal from the pH-sensitive dye, pHrodo (Supplementary Figs. 2c-f). The pHrodo label is only highly fluorescent in the acidic environment of late endosomes or lysosomes after phagocytosis, and so fluorescence from pHrodo-conjugated particles arises when they are internalized, but not surface-bound. Unlike the previous bead experiments, magnet-unbound cells also contained a small amount of IONP-conjugated myelin, indicating that cells must surpass a threshold of myelin ingestion before being captured on the magnet. Based on the enrichment achieved with simulated changes in phagocytic rate, we predicted that genome-wide screens would be highly sensitive for detecting modulators of phagocytosis.

As magnetic labeling with IONPs is highly generalizable, we also performed genome-wide screens using myelin and other well-studied model phagocytic substrates, including zymosan (yeast cell wall particles) and sheep red blood cells (RBCs) conjugated with either IgG (IgGRBC) or complement (CompRBC). We also sought to more directly address the function of particle size and charge in phagocytosis. To this end, we performed additional genome-wide screens using superparamagnetic beads of 0.43 um diameter (SmallBeads), 4.3 um diameter (BigBeads), as well as oppositely charged, cationic 1.3 um diameter beads (PosBeads). We optimized IONP label density and assay timing for each substrate (Fig. 1f, Supplementary Table 3). Because rates of uptake varied widely across substrates, the time required to achieve 50-90% of phagocytosing cells bound to the magnet varied from 30 minutes to 8 hours (see Methods, Supplementary Table 3); this was adjusted for each screen to ensure approximately equivalent selection pressure (i.e. percent of cells captured on the magnet) across screens (Supplementary Table 3). Importantly, uptake for each substrate was inhibited by cytochalasin D for both magnetized and pHrodo-labeled particles (Fig. 1f, Supplementary Fig. 2a, i-j). Furthermore, unopsonized RBCs were not readily phagocytosed, and conjugation of IONPs to unopsonized RBCs did not result in efficient uptake or magnetization of U937 cells, indicating that the IONPs themselves do not act as a phagocytosis-facilitating opsonin and are unlikely to generally skew normal uptake mechanisms for magnetized substrates (Supplementary Fig. 2 i-j).

Considerations for use of magnetic separation for genome-wide screening, and future perspective

Our screening approach was sufficiently sensitive to identify previously unrecognized regulators of phagocytosis, but the dynamic range of our genome-wide screens was somewhat low, particularly in comparison to survival screens leveraging multiple rounds of selection. We have applied a stringent FDR cutoff for hit-calling that results in a low false-positive but high false-negative rate for detection for genuine phagocytosis regulators, necessitating follow-up with sublibrary screens and single-gene validation for detection of some pathways. Enhanced sensitivity from magnetic phagocytosis screens may be achievable using a more uniform cell population, more stringent elimination of surface-bound substrates, tighter control over bound/unbound selection stringency, and optimization of phagocytic substrates to specifically engage single receptors/pathways. Some genes identified from the magnetic screen validated only in magnetic separation assays and not in pH-sensitive fluorescence-based phagocytosis assays; whether these discrepancies reflect biologically interesting differences (for example, fusion of the phagosome with the low pH lysosome vs. initial particle uptake) or idiosyncrasies of specific platforms remains to be determined, but such findings highlight the importance of orthogonal assays for investigating contributions of individual genes.

Online Methods Continued

Human monocyte-derived macrophage (MDM) generation

Leukocyte reduction system (LRS) chambers from anonymous donors were obtained from the Stanford Blood Center. PBMCs were purified as described previously[2], using gradients of Ficoll and Percoll (GE Healthcare). PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% human serum AB (Gemini Bio) for 7-10 days.

MDM phagocytosis assays

Phagocytosis assays were performed as described previously[3]. Briefly, phagocytosis substrates were labeled with pHrodo Red, SE (Thermo Fisher Scientific). Macrophages were rinsed twice with PBS and then lifted using TrypLE and cell lifters (Corning). Macrophages and substrates were co-cultured for two hours at 37°C. Phagocytosis reactions were quenched by the addition of 4°C PBS. Macrophages were identified by staining with conjugated antibodies to CD11b (clone M1/70, BioLegend). Phagocytosis was calculated as the percentage of PE+ CD11b+ macrophages and was normalized to the maximal response by each independent donor.

Human monocyte-derived macrophage genetic alterations

Genetic knockouts in primary human MDMs were performed as described previously[4]. Briefly, two sgRNA molecules targeting each of the following genetic loci, NHLRC2, NCKAPL1, TM2D3, and ELOVL1, were purchased from Synthego as modified, hybridized RNA molecules. Mature human MDMs were electroporated with Cas9 ribonuclear proteins using the P3 Primary Cell Nucleofection Kit (Lonza V4XP-3024). Macrophages were analyzed 48 hours after electroporation and in vitro phagocytosis assays were performed with biological replicates from two human donors. Indel frequencies were quantified using ICE software (Synthego).

FACS screen

U937 library cells were differentiated as above and pHrodo-labeled zymosan was applied. Cells were allowed to phagocytose for 12 hours before trypsinization and filtering as described above for magnetic screens. Cells were analyzed and sorted on a BD FACS JAZZ cell sorter. After gating for live cell singlets using FSC-Height by FSC-Area, cells were sorted based on pHrodo fluorescence intensity with 488 nm excitation and fluorescence emission detection of 530 nm ± 20 nm (530/40(488)). The sort was performed at rate of 10,000 events per second. A total of $12x10^6$ cells were sorted. The top 12% of cells with highest fluorescence and the bottom 75% of cells with the lowest fluorescence were collected and processed as above for sequencing.

RNA sequencing

Differentiated and undifferentiated U937 cells were seeded at a density of 50,000 cells per well in a 24-well plate. Two days later, cells were lysed in RLT buffer and RNA was isolated using the RNeasy Micro Kit (Qiagen). cDNA libraries were prepared and adapted for sequencing using a modified Smart-seq2 protocol [5]. In short, 10 ng of purified RNA was mixed with RNase Inhibitor, Oligo-dT30 primer, and dNTPs and heat-denatured before addition to a SuperScriptII template-switching reverse transcription reaction mixture. cDNA was amplified in a 12-cycle KAPA HiFi HotStart PCR reaction and purified using Ampure XP SPRI beads. 0.5 ng of cDNA was fragmented for sequencing using the Nextera XT DNA sample preparation kit. After tagmentaion and amplification of adapter-ligated, indexed fragments, DNA was purified using Ampure XP SPRI beads and sequenced on a NextSeq 550 (Illumina) to obtain 75 bp paired-end reads. RNA-seq data was mapped to the human genome annotation hg38 using HISAT2 version 2.0.3 (Kim et al., 2015) via the Galaxy platform resulting in 8 to 12 million mapped reads per sample. Transcript FPKM (fragments per kilobase of transcript sequence per million mapped fragments) scores were obtained using Cufflinks version 2.2.1 (Trapnell et al., 2010) with the iGenomes hg38 gtf file as a reference annotation.

Rac1/RhoA activation assay

U937 cells were grown and differentiated with 50 nM PMA for 3 days as described above. Differentiated U937 cells or RAW 264.7 cells were then plated at $1x10^6$ cells per well of a 6-well plate in normal growth medium for 1 day and changed to serum free media for an additional 24 hr before harvesting. Cell medium was then replaced with serum-free medium containing 0.0075% w/v 4.37 μm carboxyl magnetic beads or serum free media alone. Beads were allowed to settle for 10 min at room temperature then cells were placed at 37°C in a 5% CO2 incubator for 20 min. Cells were then placed on ice and washed with 1 mL ice-cold PBS to remove excess media and beads. Cells were then harvested in 200 μL ice-cold lysis buffer with a cell scraper. 20 uL was taken for protein quantification and the remaining lysate was separated into aliquots, snap frozen in liquid nitrogen, and stored at −80°C until the start of the ELISA assay. Protein concentrations were determined using the Precision Red Advanced Protein Assay that was supplied with the kits. Colorimetric G-LISA activity assay kit (Cytoskeleton, Inc., Denver, CO, cat #BK128 and #BK124) was used according to the manufacturer's instructions to quantitatively assess GTP-bound Rac1 and RhoA in U937 and RAW cells. In brief, snap frozen lysate were thawed in a room temperature water bath and protein concentrations were equilibrated using ice-cold lysis buffer. GTP-bound RhoA and Rac1 levels were then determined using the RhoA-GTP and Rac1-GTP binding 96-well plates, including a lysis buffer blank control and GTP-bound recombinant positive controls (80 pg/ml). Absorption of the ELISA wells was determined with iMark Absorbance Microplate Reader (BioRad, Hercules, CA, USA).

BioID pulldown and mass spectrometry

Large scale BioID experiments were conducted in triplicate, with each replicate consisting of differentiated U937 cells harvested from 4X 150 mm cell culture dishes at 80% confluency (~3 x 10^8 cells). U937 cells selected to stably express BirA* or NHLRC2-BirA* were differentiated with PMA as described above. The cells were incubated with 50 µM D-Biotin in culture medium for 20 hours to induce biotinylation, then washed 2X with 10 mL dPBS before lysate collection. Cells were scraped from the plate and centrifuged at 300 x g for 5 minutes, then lysed in 7X cell pellet volume of BioID Lysis Buffer (50 mM Tris pH 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 1 mM DTT, 2% (v/v) Triton X-100) supplemented with fresh protease inhibitor (cOmplete EDTA-free protease inhibitor tablet, Roche). Cells were incubated on ice with the lysis buffer for

20 minutes, then sonicated at 30% amplitude (Microson Sonicator) to shear genomic DNA. An equal volume of ice-cold 50 mM Tris, pH 7.4, was added to the lysate before centrifugation of the sample at 21,000 x g for 10 minutes at 4 ˚C. 240 µL streptavidin-coated agarose bead slurry per sample was equilibrated in 0.5X BioID Lysis Buffer and added to the clarified supernatant to tumble overnight at 4 ˚C. Agarose beads were centrifuged from the lysate at 2,500 x g for 1 minute and then subjected to harsh washing conditions. Washing proceeded with four sequential wash buffers: Wash Buffer 1 (2% SDS), Wash Buffer 2 (50 mM HEPES pH 7.5, 0.2% sodium deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA), Wash Buffer 3 (10 mM Tris pH 8.1, 250 mM LiCl, 500 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA), and Wash Buffer 4 (50 mM Tris pH 7.4, 50 mM NaCl. Each wash consisted of a 10 minute rotation in 2 mL of each wash buffer followed by a 1 minute centrifugation at 2,500 x g. After a second, final wash with 2 mL of Wash Buffer 4, beads were resuspended in 50 µL 8M Urea/50 mM ammonium bicarbonate supplemented with 4 µL 250 mM TCEP and incubated for 1 hour at 30 ˚C to reduce bound proteins. After reduction, 4 µL of 0.5M iodoacetamide was added to the agarose beads. Alkylation proceeded at room temperature for 30 minutes in the dark. The reaction volume was then diluted 8X with 50 mM ammonium bicarbonate, and 1 µg trypsin was added to digest bound proteins during an overnight incubation at 37 ˚C. The peptide-containing supernatant was harvested the next day via centrifugation (2 min, 2,500 x g). The beads were then washed with 50 uL of 50 mM ammonium bicarbonate, centrifuged, and the two supernatants were combined. Triflouroacetic acid was added to the supernatant at a final concentration of 1% (v/v) to halt digestion before MS analysis.

Following immunoprecipitation and protein digestion, protein digest was cleaned up using a Waters 1cc HLB oasis column according to manufacturer's suggested protocol. The cleaned protein digest was introduced to a Waters Liquid Chromatography column coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher). Peptides were separated using a 25 cm long and 100 µm inner diameter capillary column packed with Sepax 1.8 µm C18 resin. Peptides were eluted off in a 60 minutes gradient at flow rate of 600 nl/min from 5% to 35% Acetonitrile in 0.1% formic acid. Mass spectrometry data was acquired by one full MS scan at 120k resolution followed with MS2 using HCD at 30k resolution. The instrument was set to run in top speed mode with 3 s cycle.

Raw data was processed using Thermo Proteome Discoverer software version 2.2. MS data were searched against a human proteome database with 1% FDR at peptide level. Protein quantification was based on the precursor ion peak intensity using the label free quantitation workflow. Peptide Spectral Match (PSM) counts from three replicates of NHLRC2-BirA* BioID samples were compared against three replicates of the BirA* expression BioID control samples using the SAINT algorithm set to the default options (lowMode = 0, minFold = 1, normalize = 1) [6,7]. Fold enrichment for a given protein is calculated as the ratio of the average normalized PSM count in the NHLRC2-BirA* condition divided by the average normalized PSM count across control samples. Prior to division, the SAINT algorithm added a background factor of 1.94 to all normalized average spectral counts to prevent divide-by-zero errors.

Scanning Electron Microscopy

For SEM analysis, we used a method based on previously published work[8]. Briefly, cells cultured on 12 mm glass coverslips were fixed for 2 hr at RT with 4% formaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), rinsed in the same buffer and post-fixed for 1 hr with 1% aqueous OsO_4. After OsO_4 fixation, samples were rinsed 2X in milli-Q-H20 followed with dehydration in an ascending ethanol series (50, 70, 80, 90, 100% (2×), 5 min each). Samples were critical point dried with liquid CO_2 in a Tousimis Autosamdri-815B apparatus (Tousimis), mounted with conductive copper tape onto 15 mm aluminum stubs (Electron Microscopy Sciences), and sputter-coated with 50-100 Å of gold-palladium using a Denton DeskII Sputter Coater (Denton Vacuum). Visualization was performed with a Zeiss Sigma FE-SEM (Carl Zeiss Microscopy) operated at 5 kV, using inLens SE detection at working distance of approximately 8 mm. TIFF images were captured at 2,048 × 1,536 pixels resolution using a line averaging noise reduction algorithm.

F-actin staining

RAW 264.7 cells were plated into 1.8 cm glass coverslips the day before staining. The next day cells were fixed with 4% PFA for 10 min. Cells were then permeabilized with Triton X-100 (0.1%) for 10 min. The coverslips were washed twice with PBS. Cells were then incubated with fluorescent phalloidin (1:1,000 dilution) for 40 minutes.

Frustrated phagocytosis assay

For experiments with phalloidin staining: RAW 264.7 cells were plated to ~40% confluency in six-well plates 24 h before the experiment. 1.8 cm glass coverslips were incubated in 12 well-plates with 1 mg/ml human IgG overnight at 4°C. The day of the experiment, the IgG-coated coverslips were washed 2X with PBS and left at 4°C with PBS. The RAW 264.7 cells were washed with PBS, and then scraped in complete media. The cells in suspension were then spun down at 300Xg for 5 min. The cell pellet was resuspended in pre-warmed complete media. Simultaneously, the PBS of the IgG-coated coverslips was replaced with 500 μl of pre-warmed complete media. 500 μl of cells in suspension were added to the coverslips and spun down at 500Xg for 10 s. The 12-well plates were then incubated for 5 min at 37°C before fixation with 4% PFA for 10 min. After this, F-actin staining was performed as described before.

For live experiments with transfected cells: RAW 264.7 cells were plated to ~25% confluency in six-well plates 48 h before the experiment. 24 – 16 h before the experiment, cells were transfected LifeAct constructs using Fugene HD (Promega). From then, the procedure described above was followed until the addition of the cells to the IgG-coated coverslips. Cells were added to the coverslips and immediately placed on a confocal microscope for imaging. The objective was focused on the coverslip plane and image capture was initiated upon detection initial contact of transfected cells with the surface. Only confocal planes at the level of the coverslip were acquired.

Confocal microscopy for phalloidin staining and frustrated phagocytosis experiments

Imaging of F-actin staining experiments was performed using a spinning-disk confocal microscope (Quorum Technologies, Guelph, Canada). The system is based on an Axiovert 200M microscope (Carl Zeiss) with a 63× oil-immersion objective (numerical aperture 1.4) and a 1.5× magnifying lens. The microscope has a motorized XY stage (Applied Scientific Instrumentation, Eugene, OR) and a Piezo Z-focus drive. Images were recorded with back-thinned, cooled charge-coupled device cameras (Hamamatsu Photonics) under command of the Volocity software (version 6.2.1; PerkinElmer, Woodbridge, Canada). Extended focus projections and brightness/contrast corrections were performed with ImageJ (version 1.48; National

Institutes of Health, Bethesda, MD). Brightness/contrast parameters were adjusted across entire images and without altering the linearity of mapped pixel values. Images are representative of at least twenty determinations from three separate experiments.

Confocal microscopy for staining and imaging partially engulfed IgG beads

7.3-um hydrophobic polystyrene beads (at 0.6% w/v) were fully coated by incubation in excess BSA (at 150 μg/ml) overnight at 4C, and then washed with PBS. The BSA-coated beads (at 0.3% w/v) were subsequently fully coated by incubation in excess anti-BSA rabbit IgG (at 42.1 μg/ml) for 4 hours at room temperature, and then washed with PBS.

Control sgRNA and ELOVL1-KO RAW cells were seeded on 96-well glass bottom plates (25,000 cells per well) and incubated for 24 hours. 2.5×10^5 IgG-coated beads were added to each well. The plate was spun down at 300g for 1 min, and then incubated at 37C for 10 minutes. Cells were fixed for 20 minutes in 4% formaldehyde in PBS. The samples were washed with PBS 4 times, to remove non-adherent beads. Samples were incubated with donkey anti-rabbit AlexaFluor 647 IgG to label any exposed surfaces of IgG-beads. Nuclei were stained with Hoechst 33258. The cells were then permeabilized in 0.005% Triton X-100 in PBS and stained with tetramethylrhodamine(TMR)-phalloidin. Images were acquired by spinning disk confocal microscopy with a 100x oil objective (NA = 1.4).

Measurement of cell migration speeds

CRISPRi knockdown cell lines were generated by lentiviral infection of PLB-985 cells stably expressing SFFV-dCas9 with constructs expressing a given sgRNA along with puromycin resistance. One day after infection, we selected with (5 μg/mL) puromycin for five days. Cell cultures were then differentiated with 1.3% DMSO and 2% Nutrdioma for 6 days to achieve a neutrophil-like state. Under agarose chemotaxis assays were performed as described in Collins et al. 2015. Briefly, mCherry-H2B expressing knockdown cells were mixed with mTurquoise-H2B expressing control cells, and plated under agarose containing 100 nM Nv-fMLF, a caged derivative of the chemoattractant fMLF that can be uncaged with ultraviolet light. Cells were imaged every 30 seconds for 15 frames before gradient generation, an attractant gradient was generated and maintained by uncaging and cells were imaged for 35 additional frames. Cells were segmented and tracked using custom Matlab software. The mean cell speed before and after gradient generation was computed for both knockdown and control cells before and after gradient generation for each well. Knockdown cell speeds were normalized using the in-well control cell speeds to correct for well-to-well variability in migration conditions.

RNA extraction, reverse transcription and qPCR

Cells were harvested and total RNA was isolated using the Quick-RNA Miniprep kit (Zymo, Irvine, CA, USA). Conversion from RNA to cDNA was carried out using 5x All-In-One RT MasterMix (abm, Richmond, BC, Canada). Quantitative PCR (qPCR) was prepared with the 2x LightCycler 480 SYBR Green I Master Mix and reaction was run on a LightCycler thermal cycler (Roche). All the procedures were performed according to the instructions from the manufacturers.

Quantification and Statistical Analysis

Screen data was combined and scored using casTLE [9]. Automated quantification of phagocytosis images was performed using Incucyte ZOOM software (Essen). Unless otherwise stated, error bars represent SEM, and p-values were calculated using ANOVA followed by Dunnett's multiple comparison test to the first control group. Violin plots were generated using the Matlab Violin Plot function v1.7 with FPKM or TPM expression values extracted from published datasets [10,11]. For violin plots using mouse data, genes with non-identical nomenclature between hg38 and mm10 genome annotations were discarded. Cell plot representations of screen data were generated using Cytoscape version 3.5.1 [12] with the enhancedGraphics plugin [13], and genes were manually categorized based on known protein-protein interactions, our own clustering analysis, and published function/localization. Reactome enrichment analysis was performed by inputting all genes passing a 10% FDR in midbead genome-wide screen into the EnrichR analysis pipeline selecting the 'Reactome 2016' results. These results were sorted on adjusted P-value and select, non-redundant categories are reported along with adjusted P-values. Reactome IDs for Reactome categories reported in Figure 1 are: R-HSA-5663213, R-HSA-2029480, R-HSA-168249, R-HSA-166208, R-HSA-1660661,R-HSA-4085001.

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